THE DECOMPOSITION OF CHITIN IN AN ACID SOIL

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Introduction

A study of the distribution of fungi in a podzol soil at Delamere, Cheshire, England, has been made over the last few years (HEPPLE, 1958; PARKINSON and KENDRICK, 1960; KENDRICK and BURGES, 1962; BURGES, PARKINSON, WILLIAMS, this symposium). During these investigations, it has become apparent that the H horizon of this podzol presents a variety of unusual habitats for colonization by micro-organisms. It has been found to contain large quantities of mite faeces and fragments of dead mites and fungi (KENDRICK, 1958). It seemed important therefore to assess the ability of the soil micro-organisms to break down the chitin fraction of this debris.

To do this a modification of the cellulose film technique (TRIBE, 1957) has been used, substituting transparent strips of "chitin" (see discussion), obtained from *Sepia* shells for the cellophane used by Tribe. This technique has enabled us to study the colonization of "chitin" by various groups of micro-organisms by direct observation and to isolate chitinoclastic organisms from these decomposing strips. In addition, the principle fungi isolated from the H horizon (GAMS, 1962) have been screened for their ability to decompose chitin.

Methods

Preparation of chitin strips. Strips of chitin were removed from *Sepia* shells by treatment with 4 % hydrochloric acid. Loosely adhering cellular material was scraped off and the strips simmered in 3 % potassium hydroxide for $1\frac{1}{2}$ hours to remove more of this debris. After washing, the strips were autoclaved in tap water. Squares, 3 × 3 cm were mounted on clean flamed slides and excess moisture removed.

Burial of chitin strips. After removing large roots and twigs, soil from the H horizon was placed in glass containers, 20 × 20 × 6 cm. Ten slides bearing chitin strips were buried in each container; these containers were sealed with a glass cover. A second set of samples containing Rossi-Cholodny slides were set up as a control. The soils were incubated at 18°C for up to thirty days.

Examination of slides. At intervals, slides were recovered carefully for microscopic examination; loose soil particles were removed. After examining the general appearance of the strip, assessments of the abundance of fungi, actinomycetes, bacteria, animals and algae were made by scoring their presence or absence in fifty random quadrats on each slide. The quadrats were equal in size to the field of view using an oil immersion lens. The organisms were recognised solely by their microscopic appearance under oil, so that some misidentifications may have occurred. The results were graphed, plotting percentage occurrence of each group against time of burial.

Isolation of chitinoclastic micro-organisms. Chitin powder (B.D.H., Poole, Dorset, England) was dissolved in concentrated hydrochloric acid, filtered through glass wool and immediately reprecipitated by dilution with demineralized water. The precipitate was centrifuged, washed three times, neutralised with caustic soda and washed again. This partially hydrolysed chitin was then incorporated in the following medium (modified from that of LINGAPPA and LOCKWOOD, 1961). Chitin powder, about 10.0 g; K_2HPO_4, 1.0 g; $MgSO_4$, 1.0 g; $CaCl_2$, 0.3 g; $FeSO_4$, 0.01 g; glucosamine hydrochloride, 0.05 g; Oxoid ionagar No. 2, 8.0 g; demineralized water, 1 litre; pH 7.0. The medium was poured over a layer of solidified water agar (VELDKAMP, 1955) and the resulting plates used to isolate chitinoclastic micro-organisms and to test isolates for chitinase enzyme.

Decomposing chitin strips were cut into 2 mm squares and washed in six changes of sterile water to remove adhering soil. Six squares were placed on a plate and incubated at 25°C. Subcultures were made from squares which showed associated clearing of the medium and the isolates purified.

As a final check for their ability to break down chitin, isolates were tested on a similar medium containing unhydrolysed ground chitin.

Results

The development of micro-organisms on the chitin strips. Five chitin strips and five Rossi-Cholodny slides were examined at 2, 3, 4, 5, 6, 8 and 11 days after burial. The average percentage occurrence of the five groups of micro-organisms were estimated and plotted against time of burial (Figs. 1 and 2).

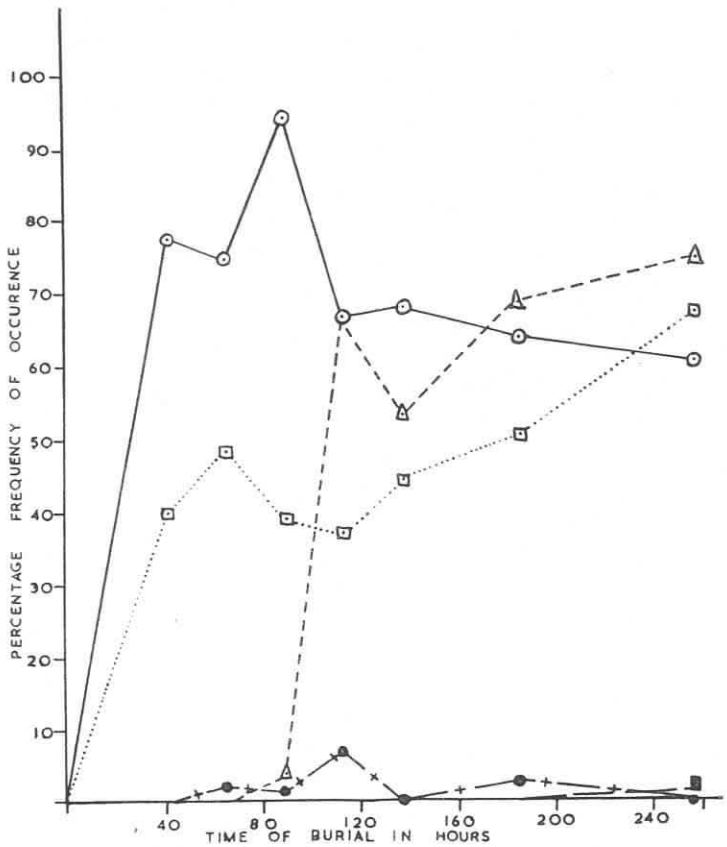

Fig. 1. Changes in the percentage frequency of occurrence of micro-organisms on "chitin" strips.

——— = bacteria; ..... = actinomycetes; - - - = fungi; + - + = algae; —— = fauna.

After two days, bacteria and actinomycetes were common on the strips. The frequency of bacteria, after rising to a maximum on the 4th day, declined slightly over the period of observation, whereas the actinomycetes became more frequent. Fungi were not apparent until the 4th day after burial, increasing rapidly after this time. After six days, fungi were found superficially and within the strip itself. Most of the bacteria were superficial, consisting of about equal numbers of rods and coccoid forms. Fungal

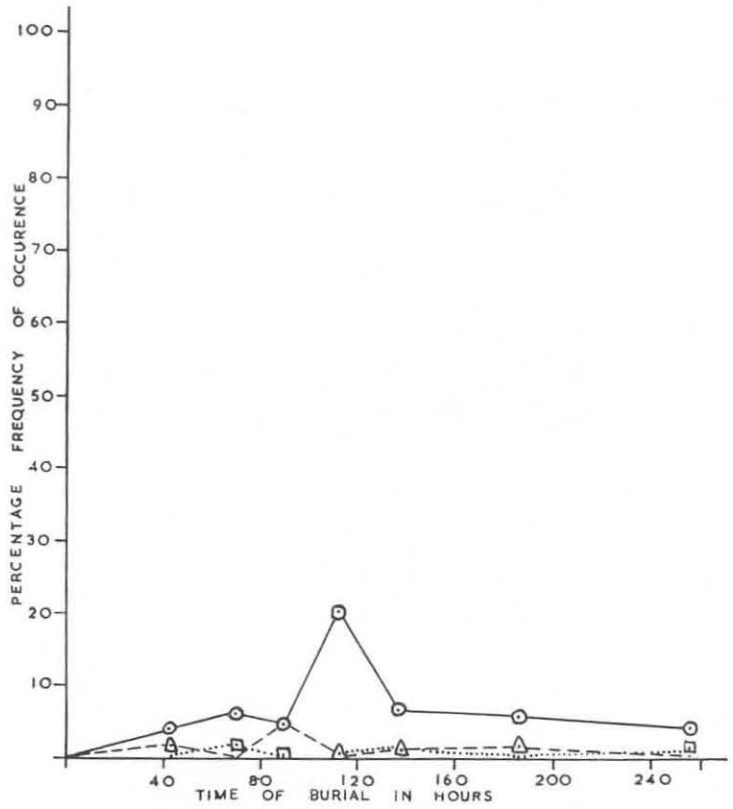

Fig. 2. Changes in the percentage frequency of occurrence of micro-organisms on Rossi-Cholodny slides.

——— = bacteria; ..... = actinomycetes; ---- = fungi.

The fauna and the algae were of low frequency.

and actinomycete spores were seen after 8 days, and usually resembled those of *Penicillium*, *Trichoderma* or mucoraceous forms. At this time the strips were becoming thin and difficult to recover. Evidence of mite action was apparent, though few mites were actually seen. Algae were equally rare.

This pattern of colonization is markedly different from that found on Rossi-Cholodny slides (Fig. 2), where the percentage occurrence of all groups was low throughout.

Substantially the same results were obtained when the experiment was repeated. On this occasion the strips were also examined after longer periods of burial (up to 19 days). After 11 days, however, the percentage occurrence of bacteria and fungi seemed comparatively stable.

Isolation of chitinoclastic micro-organisms. Small squares of decomposing chitin were plated out in the manner described, together with some unwashed

squares. Table 1 shows the species isolated after periods of 3, 7, 11, 15 and 19 days of burial. Three fungi, together with a bacterium were isolated during most of the sampling period.

TABLE 1. Chitinoclastic micro-organisms isolated from decomposing chitin strips

Isolates	Days after burial	3		7		11		15		19	
	Washing treatment	+	—	+	—	+	—	+	—	+	—
Trichoderma viride . . .		x	x	x		x	x	x	x	x	
Penicillium spinulosum . .			x	x	x		x	x		x	
Mortierella parvispora . .		x	x		x		x	x	x		
Motile, Gram-negative rod	x			x				x	x		

Chitin breakdown by other soil fungi. Fungi, probably present as mycelium on organic particles in the H horizon, were isolated by GAMS (1962) using the soil washing technique (PARKINSON and WILLIAMS, 1961). The most common of these forms are listed in Table 2, together with a record of their action on chitin. Five species were capable of attacking chitin, including *M. parvispora*, *P. spinulosum* and *T. viride* which had also been isolated from the chitin strips.

The ability of isolates to attack unhydrolyzed chitin. The chitin powder employed in the medium used to test the ability of fungi to attack unhydrolyzed chitin was of a rather course nature and tended to settle out on the bottom of the dish. The results are therefore inconclusive. However, *T. viride* and the bacterium, a Gram-negative rod, both attacked this chitin noticeably and there was some evidence that the other fungi grew best in the vicinity of the chitin particles.

Discussion

Throughout this paper, constant reference has been made to chitin strips. There is evidence, however, that this material is a chitin-protein complex

TABLE 2. The ability of fungi from the H horizon to break down chitin

Fungus	Chitin breakdown	
	6 days	20 days
Beauveria tenella	—	—
Dark sterile form	—	—
Frazeriella sp.	—	—
Fusidium sp.	+	+
Microphialophora sp. . . .	—	—
Rhinotrichum sp.?	—	—
Mortierella parvispora . . .	+	+
Oidiodendron sp.	—	—
Penicillium spinulosum . . .	—	+
Stachybotrys sp.	+	+
Trichoderma viride	+	+

(TRACEY, personal communication). Proteolytic enzymes which do not attack chitin can also break down these strips (TURNER and BERKELEY, personal communication). The colonization data must be related therefore, not to the attack of pure chitin, but to the attack of chitin as it may occur normally in soil, i.e. in combination with other nitrogenous substances. On the other hand, the strips differ from some chitin occurring in soil since there are probably few protective waxes associated with *Sepia* chitin. Thus VELDKAMP (1955) buried *Carabus* elytra in soil and found that their colonization and breakdown was slow, which contrasts with the very rapid colonization and breakdown of *Sepia* strips, encountered in this investigation. In the light of these data we can picture the speed of colonization of chitinous material in soil as being dependant upon the type and amount of associated substances, These substances may favour a vigorously growing population, with which chitin decomposing organisms must compete.

It would be difficult to assess from the direct observation of the strips, which groups of micro-organisms were responsible for chitin breakdown. It would be equally possible for the micro-organisms observed to be growing on the protein in the strips. Nevertheless, the technique is useful as a primary enrichment method, to be followed by screening of isolates for chitinoclastic activity. In the present investigation, such screening revealed that *Mortierella parvispora*, *Penicillium spinulosum* and *Trichoderma viride* were

all chitin decomposers. These fungi have already been found to be amongst the commonest forms in the H horizon (GAMS, 1962). Subsequent tests on Gams' actual isolates revealed that they too possessed chitinase enzymes. BURGES (1958) tentatively suggested, on the basis of cultural studies, that *Mortierella* spp. might be autochthonous soil fungi. The present investigation supports this conclusion. TURNER (1956) has also observed that species of *Mortierella* are often found upon natural substrates rich in organic nitrogen.

The role of actinomycetes in degrading chitin in this acid soil is uncertain. VELDKAMP (1955) reported their tremendous importance in dry agricultural soils in this respect. Whilst actinomycetes were seen on our chitin strips, they were never isolated from them, even though the isolation medium closely resembled that of LINGAPPA and LOCKWOOD (1961), specifically designed to isolate these forms. This discrepancy may be due to misidentification of actinomycetes on the strips, or to overgrowth of actinomycetes on the isolation medium by the more rapidly growing fungi, or to the inability of these particular forms to degrade chitin.

Bacteria are present only in low numbers in the H horizon, normally there being less than 1×10^6 per gram of oven dry soil. Further, many of the bacteria isolated on media of pH 7.0 are inactive at a pH below 4.0 (MATHER, 1961). The pH of the H horizon at Delamere varies between 2.8 and 3.5, so the vast development of bacteria on the chitin strips can probably be attributed to the release of ammonia and the consequent increase in pH in the vicinity of the strips. Thus the significance of the particular organism isolated in the present investigation is uncertain. From a study of its cultural behaviour it resembles *Pseudomonas chitinovorans* (VELDKAMP, 1955).

Summary

The micro-organisms involved in the breakdown of chitin in an acid soil have been investigated using a modification of Tribe's cellophane film technique. Organisms isolated from these strips included *Mortierella parvispora*, *Penicillium spinulosum* and *Trichoderma viride*. These have also been shown to be amongst the commonest fungi in the soil being studied. The significance of these and other micro-organisms is discussed.

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Discussion

G. J. F. PUGH: The saltmarsh results are interesting in confirming the high numbers of bacteria. I wonder however about the absence of Actinomycetes in these muds, because they are common in the saltmarsh muds at Gibraltar Point.

T. R. G. GRAY: I found no actinomycetes although I used the medium of Lingappa and Lockwood.

M. N. OKAFOR: The material used in this experiment is a protein-chitin complex. But I think it is important that a pure material is employed. It is possible to remove contaminating protein by treatment with a stronger solution of sodiumhydroxide than you have used. X-ray and chromatographic analyses seem to confirm that a fairly pure material can be produced. I think that it is just possible that Turner and Berkeley's enzyme in fact contained a chitinase.

T. R. G. GRAY: Rate of decomposition depends on type of associated substance. If protein is present it is rapidly colonised. A different associated substance may account for the slow decomposition of Witkamps material. Removal of protein from Sepia chitin entirely destroys the structure.

Miss J. C. WENT: On the first picture the bacteria were presumably *Cytophaga*. Veldkamp isolated on pure chitin-agar plates *Cytophaga* and many Actinomycetes from garden-soil.

W. GAMS: It is surprising to see from table 2, that *Beauveria tenella* was not chitinolytic. *Beauveria* species are well known insect parasites.

J. P. L. HARDING: You mentioned evidence of mite action on the chitin strips: does this refer to the holes observed in the strips or were pellets also observed?

T. R. G. GRAY: Any pellets found on the strips could represent contamination from the soil and do not necessarily indicate feeding by animals on the strip. A few mites were observed moving across the strips, but they were never seen in the act of feeding.

A. E. APINIS: I noticed on the slides shown, that among the fungi, actinomycetes and bacteria on the chitin strips, there were also spore-forming bacteria present. Is there any evidence that in these soils investigated fixation of nitrogen takes place? My quantitative tests of coastal Lincolnshire soils including upper reaches of salt marshes, are extremely rich in actinomycetes. Some species of these populations are even thermophilous.

T. R. G. GRAY has no information on nitrogenfixation of these soils. Actinomycetes were very abundant.

G. J. F. PUGH: It is important to realise that the more "natural chitin" is purified the more likely it is to become altered, so that chemically pure chitin may be simpler in chemical structure than "natural chitin".

J. DOEKSEN: Years ago we found, that in the end gut of a Collembolon, *Hypogastrura viatica* an obvious symbiotic bacterium is present which is capable of breaking down chitin very easily.